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Research Article

Fed-batch methanol feeding strategy for recombinant protein production by *Pichia pastoris* in the presence of co-substrate sorbitol

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Abstract

Batch-wise sorbitol addition as a co-substrate at the induction phase of methanol fed-batch fermentation by *Pichia pastoris* (Mut^+) was proposed as a beneficial recombinant protein production strategy and the metabolic responses to methanol feeding rate in the presence of sorbitol was systematically investigated. Adding sorbitol batch-wise to the medium provided the following advantages over growth on methanol alone: (a) eliminating the long lag-phase for the cells and reaching 'high cell density production' at $t = 24$ h of the process ($C_X = 70$ g CDW/l); (b) achieving 1.8-fold higher recombinant human erythropoietin (rHuEPO) (at $t = 18$ h); (c) reducing specific protease production 1.2-fold; (d) eliminating the lactic acid build-up period; (e) lowering the oxygen uptake rate two-fold; and (f) obtaining 1.4-fold higher overall yield coefficients. The maximum specific alcohol oxidase activity was not affected in the presence of sorbitol, and it was observed that sorbitol and methanol were utilized simultaneously. Thus, in the presence of sorbitol, 130 mg/l rHuEPO was produced at $t = 24$ h, compared to 80 mg/l rHuEPO ($t = 24$ h) on methanol alone. This work demonstrates not only the ease and efficiency of incorporating sorbitol to fermentations by Mut^+ strains of *P. pastoris* for the production of any bio-product, but also provides new insights into the metabolism of the methylotrophic yeast *P. pastoris*.

Keywords: ***Pichia pastoris*; fed-batch; sorbitol; methanol; oxygen; optimization**

Introduction

The methylotrophic yeast *Pichia pastoris* has become a popular host for the expression of recombinant proteins, due to its ability to produce foreign proteins at high levels using one of the strongest and most tightly regulated eukaryotic promoters, alcohol oxidase I (*AOX*1), and its facility in performing many post-translational modifications.

Since methanol is used not only as the carbon and energy source but also as an inducer of the expression of recombinant proteins, and since, at high concentrations, it inhibits growth (Zhang *et al*., 2000), fed-batch operation has been preferred. The standard protocol for the recombinant protein (r-protein) production by *P. pastoris*, through *AOX*1 promoter, is usually performed in three stages (Stratton *et al*., 1998). First, cells are grown in batches on a defined medium containing glycerol to rapidly achieve high cell densities while repressing foreign gene expression, since the *AOX*1 promoter is repressed by unlimited growth on glycerol (Tschopp *et al*., 1987). Second, glycerol is fed at limiting concentrations in order to increase biomass concentration further. This phase allows the gradual derepression of the enzymes

necessary for the dissimilation of methanol and reduces the time necessary for the cells to adapt to growth on methanol (Chiruvolu *et al*., 1997). Finally, r-protein production is induced by the addition of methanol in fed-batch mode.

Brierley *et al*. (1990) was the first group attempting the fed-batch strategy using mixed substrates of glycerol and methanol for *P. pastoris*, to increase productivity and cell density and to reduce the induction time. However, the optimal level of protein expression is not achievable with mixtures of glycerol and methanol, due to a partial repression of the *AOX*1 promoter by glycerol, which may result in lower specific productivities of r-protein (Sreekrishna *et al*., 1997; Hellwig *et al*., 2001; Xie *et al*., 2005).

On the other hand, sorbitol is a non-repressing carbon source for *AOX*1 promoter, thus sorbitol accumulation during the induction phase does not affect the expression level of r-protein. These advantages, although widely utilized for methanol utilization slow (Mut^S) strains, have recently been appreciated for methanol utilization-positive (Mut^+) strains, and have been investigated in a limited number of studies. The idea was first recommended by Sreekrishna *et al*. (1997), and the first experimental evidence showing the advantage of sorbitol supplement for the induction phase of Mut^+ strains was given by Inan *et al*. (2001), in one batch shake-flask experiment. Thereafter, Ramon *et al*. (2007) commented on the consumption mechanism of sorbitol and methanol from a batch bioreactor experiment and reported the sequential consumption of these substrates. Finally, Jungo *et al*. (2007) optimized the sorbitol content in the feed in a continuous bioreactor experiment, and performed two fed-batch bioreactor experiments, at $\mu = 0.03$/h and $\mu = 0.05$/h, using a 43% methanol:57% sorbitol C-mol : C-mol feeding ratio. The main conclusions from this study concerned the simultaneous consumption of methanol and sorbitol, it being shown that at $\mu = 0.05$/h accumulation of sorbitol did not affect the specific productivity. Moreover, other advantages, i.e. reduction in both the oxygen consumption and heat production rates in the presence of sorbitol, have been pointed out (Jungo *et al*., 2007).

Since sorbitol is a non-repressing carbon source for *AOX*1 promoter (Sreekrishna *et al*., 1997; Inan and Meagher, 2001; Jungo *et al*., 2007), there is no need to complicate the bioprocess by feeding the sorbitol fed-batch together with methanol, where below $\mu = 0.032$/h double carbon source limitations will occur, and above $\mu = 0.032$/h unconsumed sorbitol will be accumulated, as addressed by Jungo *et al*. (2007). Thus, sorbitol can easily be implemented to the existing processes, simply by adding sorbitol batchwise at the induction phase and thereby removing the restriction on the sorbitol consumption rate and the issue of double carbon source limitation. Therefore, in this study, a comprehensive study of the effects of adding sorbitol batchwise to the induction phase of recombinant human erythropoietin (rHuEPO) producing *P. pastoris* was performed. The metabolic response of *P. pastoris* Mut^+ cells to methanol feeding in the presence of sorbitol was investigated in detail, considering the production of cells, r-protein, alcohol oxidase and protease, as well as by-product formation, oxygen transfer characteristics and process yields.

Materials and methods

Microorganism and inoculum preparation

P. pastoris-E17 Mut^+ strain (Çelik *et al*., 2007) carrying EPO cDNA under the control of *AOX*1 promoter was streaked from glycerol stocks onto YPD solid medium containing 20 g/l peptone, 10 g/l yeast extract, 20 g/l glucose, 20 g/l agar and 0.1 g/l Zeocin and incubated for 48 h at 30 °C. A single colony was inoculated into 5 ml YPD liquid medium containing 100 μg/ml Zeocin, and grown shaking at 250 rpm at 30 °C overnight. The seed culture was then inoculated into BMGY medium containing 10 g/l yeast extract, 20 g/l peptone, 13.4 g/l YNB, 4×10^{-5} g/l biotin, 10 g/l glycerol and 0.1 M potassium phosphate buffer, pH 6.0, with growth allowed to proceed until C_x was ca. 2.5 g/l. Cells from the precultivation medium were collected by centrifugation and resuspended in the defined production medium.

Production media and cultivation conditions

Recombinant protein production was carried out either in laboratory-scale or pilot-scale bioreactors. Laboratory-scale batch cultivations were carried out for up to 60 h, in baffled, air-filtered $V = 250$ ml Erlenmeyer flasks containing $V_R =$ 50 ml production medium, at 250 rpm and 30 °C.

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The defined medium for laboratory-scale experiments contained 10 g/l $(NH_4)_2SO_4$, 0.1 M potassium phosphate buffer, pH 6.0, 1% v/v methanol fed every 24 h, 14.9 g/l $MgSO_4 \cdot 7H_2O$, 0.93 g/l $CaSO_4$, 4.35 ml/l filter-sterilized PTM1 salt solution, and the specified amount of sorbitol. PTM1 stock solution contained 6.0 g/l $CuSO_4 \cdot 5H_2O$, 0.08 g/l NaI, 65.0 g/l $FeSO_4 \cdot 7H_2O$, 3.0 g/l $MnSO_4 \cdot H_2O$, 20.0 g/l $ZnCl_2$, 0.5 g/l $CoCl_2$, 0.2 g/l $Na_2MoO_4 \cdot 2H_2O$, 0.02 g/l H_3BO_3, 0.3 g/l biotin and 5 ml/l concentrated H_2SO_4.

A 3.0 l pilot-scale bioreactor (Braun CT2-2) was used, having a working volume of 0.5–2.0 l and with temperature, pH, foam, stirring rate, feed inlet rate and dissolved oxygen controls. Production was carried out at N = 900 rpm, $T = 30\,^{\circ}C$ and pH 5.0, which was controlled by 25% NH_3OH. Dissolved oxygen (DO) concentration was maintained above 20% air saturation by enriching the inlet air with pure oxygen passing through a mass flow controller when needed. The defined production medium initially contained 40 g/l glycerol; 26.7 ml/l H_3PO_4 (85%), 14.9 g/l $MgSO_4 \cdot 7H_2O$, 0.93 g/l $CaSO_4$, 18.2 g/l K_2SO_4, 4.13 g/l KOH, 4.35 ml/l PTM1 salt solution and 0.1 ml/l antifoam (Y-30 Emulsion, Sigma).

A standard protocol for expression of r-proteins in *P. pastoris* under the control of the *AOX*1 promoter was followed, with slight modifications. The cells, harvested from the precultivation medium, resuspended in 10 ml sterile water and fed to the bioreactor, were first grown in batch mode until glycerol was totally consumed (phase I). Second, glycerol feed (50% glycerol containing 12 ml/l PTM1) was fed at limiting concentrations (phase II) by a predetermined exponential feeding profile, $F(t)$, calculated for a constant specific growth rate according to equation 1:

$$F(t) = \frac{\mu_0 V_0 C_{X0}}{C_{SO} Y_{X/S}} \exp(\mu_0 t) \qquad (1)$$

where μ_0 is the desired specific growth rate, V_0 is the initial volume, C_{X0} is the initial cell concentration, C_{SO} is feed substrate concentration and $Y_{X/S}$ is the cell yield on substrate. In the glycerol fed-batch phase, the pre-fixed parameters were taken as: $\mu_0 = 0.18$/h, $Y_{X/S} = 0.5$ (Cos *et al.*, 2005) and $C_{SO} = 790$ g/l.

A short transition phase of methanol pre-induction (phase III) was performed by giving a pulse of methanol feed, $C_{M0} = 1.5$ g/l (from 100% methanol containing 12 ml/l PTM1) and after 6 h r-protein production was induced by the addition of methanol in fed-batch mode (phase IV), again with a predetermined exponential feed rate given by equation 1, where the pre-fixed parameters were taken as: $\mu_0 = 0.02$/h, 0.03/h or 0.04/h, $Y_{X/S} = 0.4$ (Jungo *et al.*, 2006) and $C_{SO} = 1130$ g/l.

Four fed-batch runs were performed. In all the experiments, phases I–III were conducted in the same manner. For the constant specific growth rate, $\mu_0 = 0.03$/h, production was carried out without sorbitol, and this run was denoted as the M-0.03 condition. In the fed-batch operations with sorbitol, at the beginning of phase IV, sorbitol was added to the medium in batch mode and methanol was fed to the system at a rate calculated by equation 1 for $\mu_0 = 0.02$/h, $\mu_0 = 0.03$/h and $\mu_0 = 0.04$/h, and the conditions were denoted as MS-0.02, MS-0.03 and MS-0.04, respectively. In experiments containing sorbitol, sorbitol (450 g/l sterile stock solution) was added batchwise to the growth vessel at the end of methanol transition phase, such that $C_{S0} = 50$ g/l.

Analyses

Cell concentrations were measured using a UV-Vis spectrophotometer (Thermo Spectronic Helios-α, Waltham, MA, USA) and converted to cell dry weights (CDW) using a calibration curve obtained at 600 nm. Glycerol, methanol and sorbitol concentrations were measured by reversed-phase HPLC (Waters Alliance 2695, Milford, MA, USA) on a Capital Optimal ODS-5 μm column (Capital HPLC, Broxburn, West Lothian, UK) at 30 °C, using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.5 ml/min, and detected with a refractive index detector (Waters 2414) at 30 °C. RHuEPO concentrations were measured by high-perfomance capillary electrophoresis (HPCE; Waters, Quanta 4000E, Milford, MA, USA), as mentioned elsewhere (Çelik *et al.*, 2008) and total protein concentration was determined by the Bradford (1976) assay. SDS–PAGE was performed as described by Laemmli (1970). The gels were silver-stained using the procedure of Blum *et al.* (1987). The dynamic method (Bandyopadhyay and Humprey, 1967) was applied to find the oxygen uptake rate (*OUR*) and oxygen transfer coefficient (K_La) values. Excreted amino acid concentrations were measured using an amino acid analysis system (Waters

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HPLC, Milford, MA, USA), using the modified pico tag method (Çalık *et al*., 1998). Excreted organic acid concentrations were measured using an HPLC (Waters-2695) (İleri and Çalık, 2006). Protease activity was measured spectrophotometrically (Çalık *et al*., 1998), using 0.05 M borate buffer, pH 10, 0.05 M sodium acetate buffer, pH 5, or 0.05 M sodium phosphate buffer, pH 7. AOX activity was performed as explained by Azevedo *et al*. (2004). All analyses were performed in duplicate.

Results and discussion

Effect of initial sorbitol concentration in laboratory-scale batch cultivations

With the aim of incorporating sorbitol into the process using a Mut$^+$ *P. pastoris* strain, the effect of initial sorbitol concentration at $C_{S0} = 0$, 2, 10, 25, 40, 50, 60 and 80 g/l on cell growth and rHuEPO production was investigated in laboratory-scale cultivations. With the increasing amounts of initial sorbitol concentration, cell concentration increased to $C_{S0} = 50$ g/l, where the cell concentration ($C_X = 10$ g/l) was 1.5-fold higher than that obtained in medium containing methanol as the sole carbon source, and then decreased due to substrate inhibition (Figure 1). Thus, 50 g/l was found to be the non-inhibiting concentration limit for sorbitol. Moreover, r-protein production profiles were examined (data not shown), and a general trend of increase in rHuEPO concentration with increasing initial sorbitol concentration was observed up to $C_{S0} = 50$ g/l but then did not change significantly.

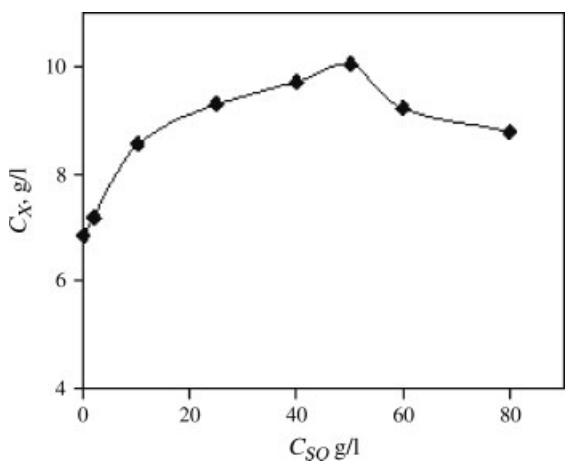

Figure 1. Variation in cell concentration with the initial sorbitol concentration

Effect of sorbitol and feeding rate of methanol on cell growth

The cell growth profiles attained in the precultivation phase of all runs were nearly the same (Figure 2). Although the process is continuous and the production (induction) phase starts at around $t = 32$ h following the precultivation period, for simplicity in calculations commencement of the production phase was taken as $t = 0$ h.

Sorbitol addition batchwise to the medium was beneficial, as it eliminated the long lag-phase ($t = 0$–9 h) for the cells and improved cell yields (Figure 2). Thus, the cells were protected from the negative effects of methanol on cell metabolism and started growing immediately in the induction medium. At $t = 24$ h, 1.7-fold higher cell concentration was obtained in medium with sorbitol (MS-0.03) compared to that without sorbitol (M-0.03). On the other hand, 'high cell density production' was achieved in as short as $t = 24$ h processes, and cell concentration reached 70 g CDW/l in MS-0.03 and MS-0.04 conditions, whereas 80 g CDW/l in M-0.03 condition was achieved in a 54 h process. These are comparable to 'high cell density fermentation' claimed for *P. pastoris*, where $C_X = 130$ g CDW/l (Wegner, 1990; Hong *et al*., 2002) achieved in 3–5 days processes.

In the M-0.03 condition, the target specific growth rate was achieved and had an average value of $\mu_0 = 0.03$/h. Comparing MS-0.03 and M-0.03 media, since the same methanol feeding profile was used, the growth rate was improved in the presence of sorbitol. The target specific growth rate $\mu_0 = 0.03$/h was selected, as the maximum specific growth rate of *P. pastoris* on methanol is $\mu_{max} = 0.14$/h, while that on sorbitol is $\mu_{max} = 0.032$/h (Jungo *et al*., 2007). Moreover, at the specific growth rates above $\mu = 0.025$/h the specific r-protein productivity was found to be independent of the growth rate (Cunha *et al*. 2004). Furthermore, high specific growth rates ($\mu > 0.038$/h) were shown to promote glycosylation (Schenk *et al*., 2008), which is an unwanted situation. Considering these, in later experiments with sorbitol, $\mu_0 = 0.02$/h and $\mu_0 = 0.04$/h conditions were also investigated.

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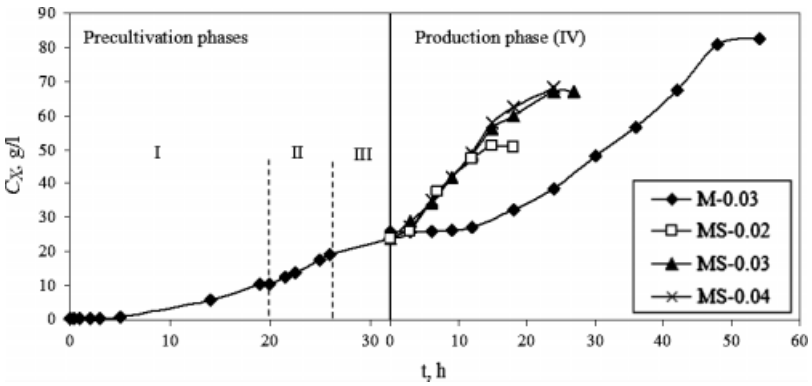

Figure 2. Variation in cell concentration with the cultivation time in the precultivation phases: glycerol batch phase (I), glycerol fed-batch phase (II), methanol transition phase (III) and production phase (IV) of the bioprocesses with different feeding profiles

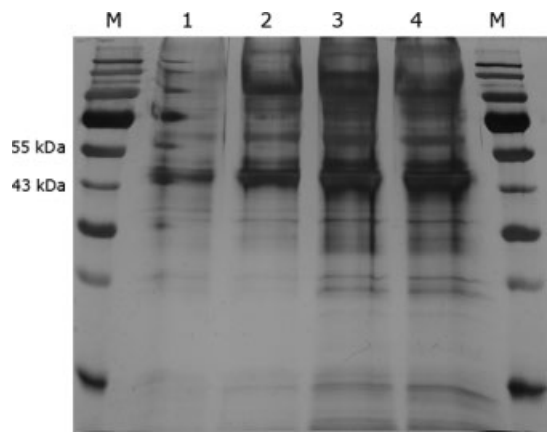

Figure 3. SDS-PAGE gel showing extracellular proteins from bioprocesses with different feeding profiles, at t = 18 h. M: Protein ladder (PageRuler™ Prestained Protein Ladder, Fermentas), Lane 1: M-0.03; Lane 2: MS-0.02; Lane 3: MS-0.03; Lane 4: MS-0.04

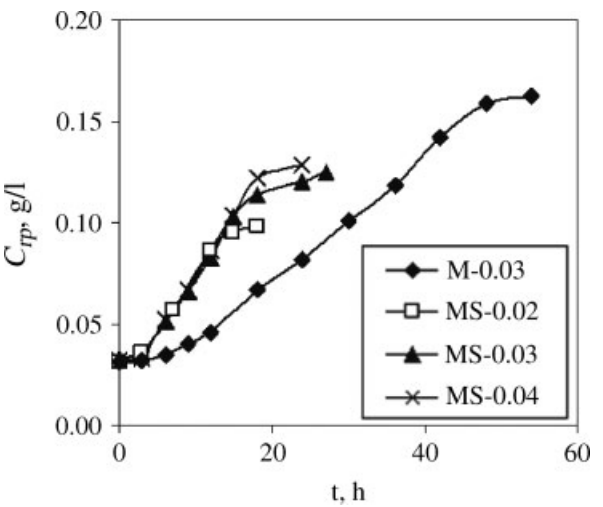

Figure 4. Variation in recombinant protein concentration with the cultivation time for different feeding profiles

Effect of sorbitol and feeding rate of methanol on recombinant protein production

The effect of sorbitol on rHuEPO production was directly observed by SDS–PAGE analysis, in which the products obtained at the same cultivation time were compared (Figure 3) and then quantified by HPCE and Bradford assay (Figure 4). At $t = 18$ h, compared to the M-0.03 condition, 1.7- and 1.8-fold higher rHuEPO was obtained in MS-0.03 and MS-0.04 conditions, respectively. Moreover, the methanol feeding rate influenced productivity as expected, since *AOX1* promoter is induced by methanol, as clearly seen when MS-0.02 and MS-0.03 media are compared in Figure 3 (lanes 2 and 3). On the other hand, the rHuEPO concentrations detected in the extracellular media from MS-0.03 and MS-0.04 experiments were nearly the same, yielding 130 mg/l rHuEPO.

Effect of methanol feeding rate on sorbitol consumption rate

The effect of methanol feeding rate on the sorbitol consumption rate was investigated. Two important findings (Figure 5) were that the methanol feeding rate did not affect the sorbitol consumption rate, and that sorbitol utilization started at $t = 0$ h in the presence of methanol and lasted for 18 h. As methanol was never detected in

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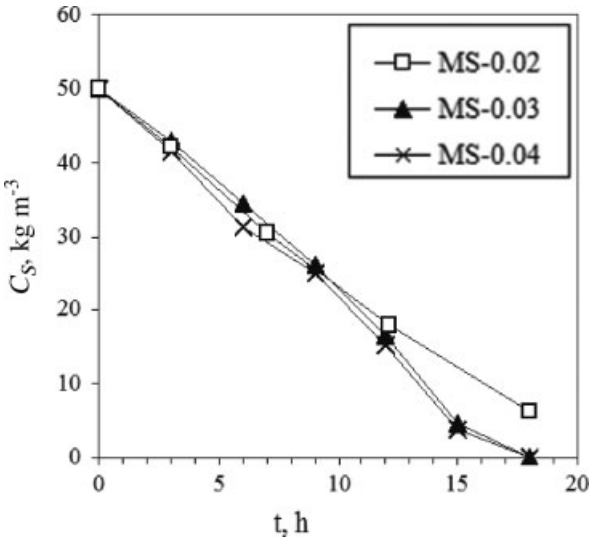

Figure 5. Variation in sorbitol concentration with the cultivation time for different feeding profiles

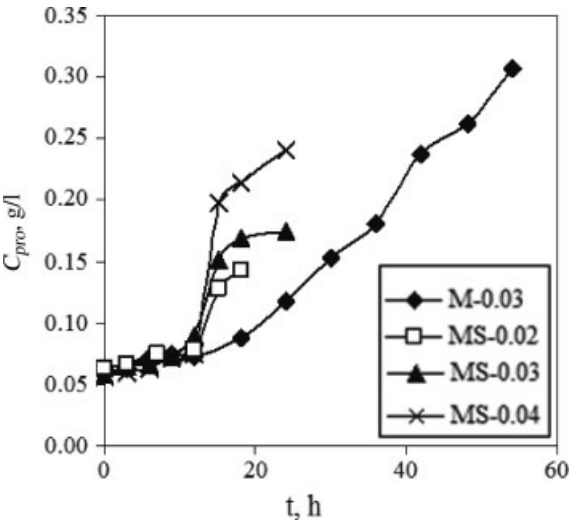

Figure 6. Variation in total protease concentration with the cultivation time for different feeding profiles

the medium, the cell consumed all methanol fed. This means that sorbitol was utilized simultaneously with methanol, confirming the results of Jungo *et al*. (2007) — rather than those of Ramon *et al*. (2007), who observed sequential utilization of sorbitol and methanol in batch cultivations of *P. pastoris*.

Effect of sorbitol and feeding rate of methanol on protease production

The extracellular activities of acidic, neutral and alkaline proteases in the *P. pastoris* production medium were measured separately and their total activity was then converted to total protease concentration. It was observed that as the methanol feed rate increased, the total protease concentration increased, in parallel to the conclusions derived by Sinha *et al*. (2005), and reaching a maximum of $C_{\text{pro}} = 0.25$ g/l in the MS-0.04 condition (Figure 6). The obvious reason is that more cells would produce more proteases. However, when the specific protease concentrations are compared at $t = 24$ h, $C'_{\text{pro}} = 2.60$ mg protease/g CDW for the MS-0.03 condition is much less than $C'_{\text{pro}} = 3.06$ mg protease/g CDW for the M-0.03 condition. Thus, it can be concluded that the presence of sorbitol reduces the specific protease production. Moreover, it was observed that while the neutral proteases were almost the same for all conditions, twice as much alkaline protease was observed in the MS-0.04 condition compared to other conditions. Thus, alkaline proteases were affected the most by the feeding rate of methanol.

Effect of sorbitol and feeding rate of methanol on alcohol oxidase production

As rHuEPO production is coupled to alcohol oxidase (AOX) production, induced by the presence of methanol, variations in AOX activity with the cultivation time were determined and are given in Figure 7. The specific AOX activity results showed a linear relationship to increasing feeding rates of methanol; thus, at MS-0.04 condition the highest specific activity was obtained as 82 U/g CDW and the highest specific activities in the M-0.03 and MS-0.03 conditions were the same at $t = 6$ h (Figure 7). The enzyme had its highest activity at $t = 6$ h of the bioprocess in all conditions, and thereafter decreased. The decrease in AOX-specific activity was larger for sorbitol-containing media, due to their higher cell concentration and lower specific methanol consumption rates compared to M-0.03 conditions. Moreover, the specific AOX activity obtained for methanol-containing medium was similar in profile but slightly higher than that obtained in a batch induced fermentation study using complex medium, where methanol was again the sole carbon source (Ramchuran *et al*., 2005).

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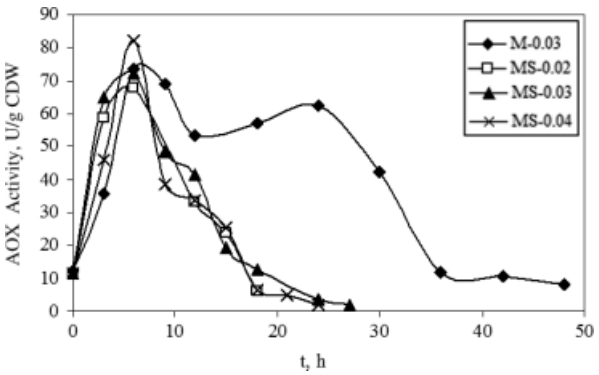

Figure 7. Variation in alcohol oxidase activity with the cultivation time for different feeding profiles

Effect of sorbitol and feeding rate of methanol on organic and amino acid concentration profiles

Lactic acid (Lac) was detected as a metabolic by-product in highest amounts in the medium without sorbitol, $C_{LA} = 2.6$ g/l, whereas in media with sorbitol, only $C_{LA} = 0.3$ g/l was produced in all the conditions. Lactic acid is the main metabolic product formed in the case of insufficient oxygen, when the TCA cycle cannot take place efficiently. Since sorbitol was proposed to be used to lower the oxygen requirement of *P. pastoris*, the results point to the fact that this target was achieved. Moreover, shortening of the process period with sorbitol supplementation eliminated the lactic acid build-up period observed after $t = 25$ h of the bioprocess.

The other organic acids detected in the medium were mostly the TCA cycle metabolites, i.e. citric acid, α-ketoglutaric acid, fumaric acid and succinic acid. As a general trend, more of these metabolites were secreted into the extracellular medium under the M-0.03 conditions. Again, this is related to the fact that, in the presence of sorbitol, there is increased flux although the TCA cycle worked better. Since formaldehyde is readily oxidized to formic acid or goes into the assimilatory pathway and enters glycolysis, it was never detected in the medium. Formic acid was detected in the medium and increased with increasing methanol feed rate. On the other hand, the formic acid concentration in the extracellular medium was much higher in the medium without sorbitol, reaching $C_{for} = 0.15$ g/l, meaning that formaldehyde was dissimilated more, rather than entering the glycolytic pathway, as occurs in the absence of the co-substrate sorbitol. Ethanol or acetate was never detected in HPLC analyses, supporting the results of other studies (Jungo *et al*., 2006, 2007).

In all the studied conditions, the alanine family of amino acids [alanine (Ala), leucine (Leu), valine (Val)] were never detected in the medium. This is probably due to the high Ala and Leu content of EPO, which contains 13% Ala and 11% Leu, whereas the other amino acids are present at an average level of 4.2%. The other possible reason is that both these amino acids are synthesized from pyruvate, and pyruvate is a central and highly controlled node in metabolism. Moreover, cysteine was detected only in sorbitol-containing media. Aspartic acid (Asp) was the amino acid secreted in highest amounts in all conditions, roughly $C_{Asp} = 0.1$ g/l. The highest total amount of amino acid excreted to the extracellular medium did not vary according to the presence of sorbitol but, rather, according to the methanol feed rate, reaching a maximum of 0.54 g/l. For the production of proteins, most microorganisms have the metabolic machinery to synthesize all essential amino acids from simple carbon and nitrogen sources; and regulation of the metabolic reaction network should cause good coupling of supply and demand for the amino acids. If the supply exceeds the demand, the amino acids would be excreted to the extracellular medium, and if they are required again, the amino acids could be re-absorbed from the medium. Moreover, the secreted proteins in the extracellular medium are degraded by the extracellular proteases, thus increasing the amino acid concentration in the medium.

Oxygen transfer characteristics of the bioprocess

During rHuEPO production by *P. pastoris* in fed-batch culture using different methanol feeding rates, the dynamic method was applied to determine the variations in volumetric mass transfer coefficient (K_La), oxygen uptake rate (OUR), oxygen transfer rate (OTR), maximum oxygen utilization rate (OD), Damköhler number (Da) and effectiveness factor (η). The values of these parameters are given in Table 1.

K_La first increased and then decreased with the cultivation time (Table 1). K_La depends on agitation rate, temperature, rheological properties of

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Table 1. The variations in oxygen transfer parameters with different methanol feeding profiles

Profile code	t (h)	K_La (s^{-1})	$OTR \times 10^3$ (mol/m^3/s)	$OTR_{max} \times 10^3$ (mol/m^3/s)	$OUR \times 10^3$ (mol/m^3/s)	$OD \times 10^3$ (mol/m^3/s)	*Da*	η
	3	0.022	16.1	23.1	14.3	—	—	—
	6	0.043	31.5	45.1	29.7	1084.2	24.1	0.03
	12	0.067	58.7	83.8	47.3	602.2	8.6	0.08
M-0.03	18	0.036	26.4	37.7	24.5	123.7	3.3	0.20
	24	0.022	16.1	23.1	14.3	76.7	3.3	0.19
	30	0.061	44.7	63.9	42.9	210.1	3.3	0.20
	36	0.032	23.5	33.5	21.6	101.5	3.0	0.21
	42	0.044	32.3	46.1	30.4	155.3	3.4	0.20
	48	0.044	32.3	46.1	30.4	153.2	3.3	0.20
	1	0.029	8.2	10.2	8.0	89.2	8.7	0.09
	7	0.038	10.2	12.7	10.0	28.1	2.2	0.36
MS-0.02	13	0.017	6.9	8.6	6.5	25.6	3.0	0.26
	15	0.018	6.3	7.9	6.0	71.8	9.1	0.08
	18	0.016	6.6	8.3	6.3	—		
	6	0.022	11.3	11.8	10.9	24.4	2.1	0.45
MS-0.03	12	0.043	22.0	27.5	21.6	65.1	2.4	0.33
	18	0.043	22.0	27.5	21.6	146.9	5.3	0.15
	24	0.014	7.2	9.0	6.8	82.6	9.2	0.08
	2	0.034	13.4	16.8	13.1	34.2	2.0	0.38
MS-0.04	9	0.045	23.4	29.2	23.0	59.3	2.0	0.39
	12	0.050	23.1	28.8	22.7	61.6	2.1	0.37
	18	0.035	18.7	23.4	18.3	105.1	4.5	0.17
	21	0.033	15.5	19.4	15.1	145.8	7.5	0.10

the fermentation medium and the presence of fine particles in the mass transfer zone. Temperature and agitation rate were kept constant throughout the bioprocess, therefore the changes in K_La are likely to be due to the change in rheological properties of the fermentation medium and the presence of fine particles in the mass transfer zone. As the cells grow, they secrete metabolites and proteins to the extracellular medium, which results in a resistance zone for mass transfer. Moreover, *P. pastoris* is an organism capable of high cell density fermentation, thus cell concentration reaching 80 g/l and causing cell coalescence should have a major impact on mass transfer. On the other hand, higher cell concentration causes higher oxygen uptake rate, which affects the volumetric mass transfer coefficient positively. The interaction of these two parameters (mass transfer resistance and *OUR*) determines the profile of K_La values throughout the fermentation process. The K_La values were generally highest at $t = 12$ h of the bioprocess, and had values of $K_La = 0.067$/s, 0.017/s, 0.043/s and 0.050/s for the M-0.03, MS-0.02, MS-0.03 and MS-0.04 conditions, respectively. The K_La values obtained were in the lower range of previously reported values for *P. pastoris* fermentations (0.01–0.3/s; Cunha *et al*., 2004; Zhang *et al*., 2005; Isett *et al*., 2007), probably due to the dimensions of the bioreactor used.

The oxygen uptake rate (*OUR*), which depends on the metabolic functions and growth phases of the microorganism, tended to increase at the start of the production process, due to the increase in biomass production rate, the biomass concentration and the substrate consumption rate. The *OUR* was at its maximum at $t = 7$–12 h of the bioprocess, having values of $OUR = 47.3$ mmol/m^3/s, 10.0 mmol/m^3/s, 21.6 mmol/m^3/s and 23.0 mmol/m^3/s, respectively, for the M-0.03, MS-0.02, MS-0.03 and MS-0.04 conditions, and thereafter decreased (Table 1). The obtained *OUR* values fell within the range of various *OUR* values reported previously (15–250 mmol/m^3/s) for different *P. pastoris* fermentation strategies (Charoenrat *et al*., 2006; Hu *et al*., 2008). Remarkably, the presence of sorbitol appeared to lower the *OUR*, and increasing methanol feed rates caused an increase in the

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OUR, in relation to the increase in K_La values. The *OTR* showed a similar trend to *OUR* but was naturally higher than *OUR*, since the control system acted to maintain a dissolved oxygen concentration (DO) >20% throughout the bioprocess.

The maximum possible oxygen utilization rate ($\mathrm{OD} = \mu_{\max} C_X / Y_{X/O}$) and the maximum possible mass transfer rate ($OTR_{\max} = K_L a C_0^*$) were also calculated throughout the growth phase of the bioprocess. A kind of Damköhler number, *Da*, defined as maximum possible oxygen utilization rate per maximum mass transfer rate (Çalık *et al*., 2000), and effectiveness factor, η, defined as the oxygen uptake rate per maximum possible oxygen utilization rate values (Çalık *et al*., 2000), can be seen in Table 1; where $\eta = 1$ is the ideal condition. It is clear from Table 1 that mass-transfer resistances are effective ($Da \gg 1$) at all times, rather than being biochemical reaction-limited ($Da < 1$), with the highest, $Da = 24.1$, obtained in the M-0.03 condition, as expected. In this condition the cells had high oxygen demands (OD), whereas the maximum possible oxygen transfer rates ($OTR_{\max}$) were comparatively much lower. Table 1 shows that the effectiveness factor (η) remained low at all times, indicating that the cells are consuming less oxygen than the oxygen demanded (OD). The relatively higher η values obtained in media containing sorbitol show that, under these conditions, oxygen uptake rates approached the maximum demanded by the cells' metabolism. Moreover, the highest η value was obtained for the MS-0.03 condition, $\eta = 0.45$.

Yield coefficients and specific production rates of the bioprocess

The overall yield of cells ($Y_{X/S}$) and product ($Y_{P/S}$) formed per mass of substrate consumed, as well as the overall yield of product formed per mass of cells generated ($Y_{P/X}$), were calculated for the rHuEPO production processes with different feeding profiles; considering the whole process in each case (Table 2). For the calculation of yield coefficients related to substrate, both sorbitol and methanol were considered, since both are used as carbon sources, therefore denoted by s'.

The highest overall product yield on cell ($Y_{P/X} =$ 1.97 mg/g) was obtained in the medium without sorbitol (M-0.03 condition). On the other hand, using sorbitol together with low feeding rates of the toxic methanol (MS-0.02 condition), instead of using methanol alone (M-0.03 condition), increased the overall product yield on the total carbon source ($Y_{P/S'} = 1.15$ mg/g) two-fold. Comparing the M-0.03 and MS-0.03 conditions, 1.4-fold higher $Y_{P/S'}$ was obtained in sorbitol-containing medium. Moreover, the highest cell yields on substrate ($Y_{X/S'} =$ 0.60 g/g) were obtained at MS-0.02 condition, which is comparable with other yeasts and filamentous fungi, where $Y_{X/S}$ is typically in the range 0.4–0.8 g/g, having a value of $Y_{X/S} = 0.50$ g/g for *S. cerevisiae* grown aerobically on glucose (Bailey and Ollis, 1986).

Table 2. Overall yield coefficients

Profile code	$Y_{P/X}$ (mg/g)	$Y_{P/S'}$ (mg/g)	$Y_{X/S'}$ (g/g)
M-0.03	1.97	0.59	0.30
MS-0.02	1.92	1.15	0.60
MS-0.03	1.87	0.85	0.45
MS-0.04	1.88	0.73	0.39

The variation in total specific growth rate (μ_t), specific oxygen uptake rate (q_o), specific recombinant product formation rate (q_{rp}) and specific sorbitol consumption rate (q_s) throughout the rHuEPO production processes with different feeding profiles are given in Table 3. Although a specific growth rate (μ_0) is aimed in fed-batch processes, it is obtained on average, but not strictly constant at the desired value. Thus, for the M-0.03 condition where $\mu_0 = 0.03$/h was aimed for, the average of specific growth rates obtained throughout the process was 0.03/h. Thereafter, adding sorbitol batchwise to the system without changing the feed rate evidently resulted in higher specific growth rates (Table 3), partly due to methanol (μ_0) and partly due to sorbitol (μ_S).

The specific sorbitol consumption rate was the highest at the beginning of the process and then decreased. Sorbitol was consumed at the highest rate in MS-0.04 condition, where $q_s = 0.107$ g/g/h (Table 3). Thus, the feeding rate of methanol also affects the sorbitol consumption rate, likely due to the speeding up of the metabolism. On the other hand, the highest specific oxygen uptake rate was obtained ($q_o = 0.2$ g/g/h) in the medium without sorbitol (M-0.03), as expected, and moreover the q_o values increased with increasing methanol feed rate (Table 3). The methanol utilization pathway requiring oxygen is again the reason behind

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Table 3. Variation in specific rates throughout the bioprocesses

Profile code	t (h)	μ_t (h^{-1})	q_s (g/g/h)	q_o (g/g/h)	q_{rp} × 1000 (g/g/h)
	3	0.005		0.064	0.029
	6	0.008		0.131	0.060
	9	0.012			0.075
	12	0.029		0.199	0.113
M-0.03	18	0.034		0.087	0.101
	24	0.040		0.043	0.088
	30	0.039		0.103	0.080
	36	0.037		0.044	0.079
	42	0.040		0.052	0.070
	48	0.027		0.043	0.043
	54	0.017			0.036
	3	0.078	0.104	0.038	0.148
	7	0.065	0.067	0.031	0.148
MS-0.02	12	0.039	0.068	0.016	0.107
	15	0.015	0.061	0.014	0.047
	18	0.001	0.028	0.014	0.020
	3	0.064	0.083		0.112
	6	0.066	0.078	0.017	0.152
	9	0.058	0.069		0.131
MS-0.03	12	0.054	0.073	0.051	0.134
	15	0.041	0.049		0.100
	18	0.026	0.026	0.041	0.047
	24	0.018	0.000	0.009	0.032
	27	0.007	0.000		0.029
	3	0.072	0.107	0.058	0.129
	6	0.074	0.074		0.172
	9	0.063	0.059	0.063	0.145
MS-0.04	12	0.061	0.069	0.044	0.137
	15	0.047	0.044		0.118
	18	0.028	0.020	0.034	0.060
	24	0.025	0.000	0.026	0.036

the above observations. Moreover, the specific oxygen uptake rate values obtained in this study were generally in agreement with the q_o range (0.02–0.05 g/g/h) obtained in *P. pastoris* fermentations with various glycerol fed-batch strategies investigated in a defined medium (Hu *et al*., 2005).

More importantly, the highest specific r-protein production rate was obtained in the MS-0.04 condition as $q_{rp} = 0.172$ mg/g/h at $t = 6$ h, compared to $q_{rp} = 0.113$ mg/g/h in the M-0.03 condition (Table 3). It was logical to obtain increased q_{rp} with increased methanol feeding rates, since r-protein production is coupled to AOX enzyme in the methanol utilization pathway, and the highest specific AOX activity was also obtained at $t = 6$ h of the bioprocess. On the other hand, q_{rp} towards the end of the process did not differ significantly with the feeding rate of methanol, or μ_0 in this case. This is congruent with the findings of Schenk *et al*. (2008), who reported that the specific productivity of a Mut^+ *P. pastoris* strain increases strongly with the specific growth rate for $\mu = 0$–0.02/h, and increases only slightly with the specific growth rate above this limit.

Conclusion

Since sorbitol is a non-repressing carbon source for *AOX*1 promoter, adding sorbitol batchwise as a co-substrate at the induction phase of *P. pastoris* fermentation and the metabolic responses to methanol feeding rate in the presence of sorbitol were investigated in detail. Since sorbitol and methanol were utilized simultaneously, and the methanol feeding rate did not affect the sorbitol consumption rate, adding sorbitol batchwise to the medium was beneficial and the strategy in turn eliminated the long lag-phase ($t = 0$–9 h) for the culture, 'high cell density production' being achieved at $t = 24$ h of the process, reaching $C_X = 70$ g CDW/l in the MS-0.04 condition.

In the medium with sorbitol (MS-0.03), 1.7-fold higher cell (at $t = 24$ h) and rHuEPO (at $t = 18$ h) concentrations were obtained compared to that without sorbitol (M-0.03). The highest specific r-protein production rate was obtained at $t = 6$ h ($q_{rp} = 0.172$ mg/g/h) at MS-0.04 condition, as a consequence of the maximum specific AOX activity being obtained at $t = 6$ h. Sorbitol supplementation reduced the specific protease production after $t = 12$ h, and also eliminated the lactic acid build-up period.

The presence of sorbitol lowered the *OUR* two-fold; however, increasing methanol feed rates caused an increase in the *OUR*. The relatively higher η values obtained in media containing sorbitol show that the maximum possible oxygen utilization (OD) values were better approached. Using sorbitol together with low feeding rates of methanol (MS-0.02 condition), instead of using methanol alone (M-0.03 condition), increased the overall product yield on the total carbon source ($Y_{P/S'} = 1.15$ mg/g) two-fold. Comparing the M-0.03 and

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MS-0.03 conditions, 1.4-fold higher $Y_{P/S'}$ value was obtained in the sorbitol-containing medium.

Considering all the benefits that accrue from incorporating sorbitol into the medium batchwise for fermentations with a Mut$^+$ strain of *P. pastoris*, more economic and efficient industrial processes would appear to be an inevitable consequence of its use. Our study contributes not only to developing design strategies that can be applied for the production of any bio-product using *P. pastoris*, which has become a popular microbial production system in the last decade, but also to further understanding the metabolism of this methylotrophic yeast.

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References

Azevedo AM, Cabral JMS, Prazeres DMF, *et al*. 2004. Thermal and operational stabilities of *Hansenula polymorpha* alcohol oxidase. *J Mol Catal B Enzymol* **27**: 37–45.

Bailey JE, Ollis DF. 1986. *Biochemical Engineering Fundamentals*. McGraw Hill: Singapore.

Bandyopadhyay B, Humprey AE. 1967. Dynamic measurement of the volumetric oxygen transfer coefficient in fermentation systems. *Biotechnol Bioeng* **9**: 533–544.

Blum H, Beier H, Gross HJ. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**: 93–99.

Bradford MM. 1976. A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* **72**: 248–254.

Brierley RA, Bussineau C, Kosson R, *et al*. 1990. Fermentation development of recombinant *Pichia pastoris* expressing the heterologous gene: bovine lysozyme. *Ann NY Acad Sci* **589**: 350–362.

Charoenrat T, Ketudat-Cairns M, Jahic M, *et al*. 2006. Increased total air pressure versus oxygen limitation for enhanced oxygen transfer and product formation in a *Pichia pastoris* recombinant protein process. *Biochem Eng J* **30**: 205–211.

Chiruvolu V, Cregg JM, Meagher MM. 1997. Recombinant protein production in an alcohol oxidase-defective strain of *Pichia pastoris* in fed-batch fermentations. *Enzyme Microb Tech* **21**: 277–283.

Cos O, Resina D, Ferrer P, *et al*. 2005. Heterologous production of *Rhizopus oryzae* lipase in *Pichia pastoris* using the alcohol oxidase and formaldehyde dehydrogenase promoters in batch and fed-batch cultures. *Biochem Eng J* **26**: 86–94.

Cunha AE, Clemente JJ, Gomes R, *et al*. 2004. Methanol induction optimization for scFv antibody fragment production in *Pichia pastoris*. *Biotechnol Bioeng* **86**: 458–467.

Çalık P, Çalık G, Özdamar TH. 1998. Oxygen transfer effects in serine alkaline protease fermentation by *Bacillus licheniformis*: use of citric acid as the carbon source. *Enzyme Microb Tech* **23**: 451–461.

Çalık P, Çalık G, Özdamar TH. 2000. Oxygen transfer strategy and its regulation effects in serine alkaline protease production by *Bacillus licheniformis*. *Biotechnol Bioeng* **69**: 301–311.

Çelik E, Çalık P, Halloran SM, Oliver SG. 2007. Production of recombinant human erythropoietin from *Pichia pastoris* and its structural analysis. *J Appl Microbiol* **103**: 2084–2094.

Çelik E, Özbay N, Oktar N, Çalık P. 2008. Use of biodiesel by-product crude glycerol as the carbon source for fermentation processes by recombinant *Pichia pastoris*. *Ind Eng Chem Res* **47**: 2985–2990.

Hellwig S, Emde F, Raven NPG, *et al*. 2001. Analysis of single chain antibody production in *Pichia pastoris* using on-line methanol control in fed-batch and mixed-feed fermentation. *Biotechnol Bioeng* **74**: 344–352.

Hong F, Meinander NQ, Jonsson LJ. 2002. Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*. *Biotechnol Bioeng* **79**: 438–449.

Hu XQ, Chu J, Zhang Z, *et al*. 2008. Effects of different glycerol feeding strategies on *S*-adenosyl-l-methionine biosynthesis by PGAP-driven *Pichia pastoris* overexpressing methionine adenosyltransferase. *J Biotechnol* **137**: 44–49.

Inan M, Meagher MM. 2001. Non-repressing carbon sources for alcohol oxidase (AOX1) promoter of *Pichia pastoris*. *J Biosci Bioeng* **92**: 585–589.

Isett K, George H, Herber W, Amanullah A. 2007. Twenty-four-well plate miniature bioreactor high-throughput system: assessment for microbial cultivations. *Biotechnol Bioeng* **98**: 1017–1028.

İleri N, Çalık P. 2006. Effects of pH strategy on endo- and exo-metabolome profiles and sodium potassium hydrogen ports of β-lactamase producing *Bacillus licheniformis*. *Biotechnol Progr* **22**: 411–419.

Jungo C, Rerat C, Marison IW, von Stockar U. 2006. Quantitative characterization of the regulation of the synthesis of alcohol oxidase and of the expression of recombinant avidin in a *Pichia pastoris* Mut$^+$ strain. *Enzyme Microb Tech* **39**: 936–944.

Jungo C, Schenk J, Pasquier M, *et al*. 2007. A quantitative analysis of the benefits of mixed feeds of sorbitol and methanol for the production of recombinant avidin with *Pichia pastoris*. *J Biotechnol* **131**: 57–66.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Ramchuran SO, Mateus B, Holst O, Karlsson EN. 2005. The methylotrophic yeast *Pichia pastoris* as a host for the expression and production of thermostable xylanase from the bacterium *Rhodothermus marinus*. *FEMS Yeast Res* **5**: 839–850.

Ramon R, Ferrer P, Valero F. 2007. Sorbitol co-feeding reduces metabolic burden caused by the overexpression of a *Rhizopus oryzae* lipase in *Pichia pastoris*. *J Biotechnol* **130**: 39–46.

Schenk J, Balazs K, Jungo C, *et al*. 2008. Influence of specific growth rate on specific productivity and glycosylation of a

recombinant avidin produced by a *Pichia pastoris* Mut$^+$ strain. *Biotechnol Bioeng* **99**: 368–376.

Sinha J, Plantz BA, Inan M, Meagher MM. 2005. Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast *Pichia pastoris*: case study with recombinant ovine interferon-τ. *Biotechnol Bioeng* **89**: 102–112.

Sreekrishna K, Brankamp RG, Kroop KE, *et al*. 1997. Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. *Gene* **190**: 55–62.

Stratton J, Chiruvolu V, Meagher M. 1998. High cell-density fermentation. In *Methods in Molecular Biology: Pichia Protocols*, Higgins DR, Cregg JM (eds). Humana Press: Totowa, NJ; 107–120.

Tschopp JF, Brust PF, Cregg JM, *et al*. 1987. Expression of the *lacZ* gene from two methanol-regulated promoters in *Pichia pastoris*. *Nucleic Acids Res* **15**: 3859–3876.

Wegner G. 1990. Emerging applications of the methylotrophic yeasts. *FEMS Microbiol Rev* **7**: 279–283.

Xie J, Zhou Q, Du P, Gan R, Ye Q. 2005. Use of different carbon sources in cultivation of recombinant *Pichia pastoris* for angiostatin production. *Enzyme Microb Tech* **36**: 210–216.

Zhang W, Inan M, Meagher MM. 2000. Fermentation strategies for recombinant protein expression in the methylotrophic yeast *Pichia pastoris*. *Biotechnol Bioproc Eng* **5**: 275–287.

Zhang W, Li ZJ, Agblevo FA. 2005. Microbubble fermentation of recombinant *Pichia pastoris* for human serum albumin production. *Process Biochem* **40**: 2073–2078.

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